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Research Paper

The effect of *Foeniculum vulgare* essential oil on lead and manganese co-exposure induced changes in bone marrow of rats during development

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Abstract

The exposure to toxic levels of lead and manganese during developmental period can impair growth with potential adverse effects on biological tissues. In the current study, we examined the toxicity effects of lead and manganese maternal ingestion in drinking water during gestation and lactation on blood and bone marrow changes. Wistar rat pups via their pregnant dams were given distilled water, 4.79 mg/ml Mn and 0.2 mg/ml Pb in drinking water during prenatal and post natal until weaning. After that, the co-exposed rats to lead and manganese received injections of essential oil of *Foeniculum vulgare* (0.1 ml/kg) for 21 days. The results showed that metal-exposed pups had lower body and bone weights and elevated blood concentrations of the respective metal. The obtained results showed also that lead and manganese intoxication resulted in a significant decrease in red blood cells count, hemoglobin concentration, % packed cell volume and mean cell volume. Moreover, blood smears examined illustrate that lead and manganese induce disturbances in the development of different types of red blood cell during development by an increase in acanthocytes and presence dacaryocytes. Regarding the histopathological examination, metal co-exposed induced a rarification of the bone marrow and a regression of the erythroblastosis. However, treatments with *Foeniculum vulgare* essential oil improved the majority of hematological parameters and reduced the alterations of histopathological damage in bone marrow.

Keywords: Fennel essential oil, GC/MS, Heavy metals, Haematological parameters, Bone marrow.

Introduction

Manganese (Mn) is one of the important heavy metals that are widely distributed in the environment, including water, air and soil, and can eventually enter the human body through the food chains[1]. Thus, Mn-induced osteotoxicity as a result of chronic Mn exposure in humans as well as animals deserves further in-depth investigation. From studies related to chronic Mn poisoning, it was presumed that large amounts of Mn caused depression of both erythropoiesis and granulocyte formation[2].

Lead (Pb) is another important heavy metal that is broadly used in the industrial field. It has been shown that Pb can induce a broad range of effects on many organ systems in humans such as a disturbance hemoglobin synthesis, influence behavior, and the neurological system in children[3]. The bone is the main organ in which Pb is accumulated, and more than 90% of Pb is found in the bone[4]. After absorption, most Pb binds to proteins in erythrocytes and is distributed to soft tissues and bone,

the latter is the main depot for this metal[5]. Some authors suspected that exposure to Pb may be related to bone marrow damage in rats[6]. A few studies have demonstrated that Pb may interfere with bone formation and bone strength and may increase the risk of osteoporosis and bone fracture[7]. Some experimental studies have also suggested that Pb may affect osteoblast and osteoclast function[8]. The exposure to chemical mixtures is a common and important determinant of toxicity. It receives concern for their introduction by ingestion and inhalation. However, few in vivo mixture studies have been conducted to understand the health effects of chemical mixtures compared with single chemicals.

Foeniculum vulgare Mill. (fennel) is a biennial medicinal and aromatic plant belonging to the apiaceae family (Umbelliferae). It is generally considered as indigenous to the shores of Mediterranean Sea, but has become widely naturalised in many parts of the world[9]. Pharmacologically, the fennel essential oil (FEO) has been shown reported to exhibit antifungal, anti-inflammatory, analgesic, antiosteoporotic, antithrombotic, hepatoprotective, anti-diabetic, antimutagenic, antioxidant and anti-bacterial biological effects[10-12]. The aim of the present study, accordingly, is to evaluate the fennel essential oil protective effect against Mn and Pb toxicity in the blood and the bone marrow of rats during gestation and lactation.

Materials and methods

Plant materiel and extraction

Dried *Foeniculum vulgare* Mill. (fennel) seeds were purchased from local herb market in Saida (Algeria) and were identified and authenticated by an expert taxonomist. We used 50 g of dried seeds *Foeniculum vulgare* that were processed by steam distillation, over a period of four hours, in an all-glass apparatus[13].

Determination of the chemical composition of the FEO by GC/MS

The gas chromatography-mass spectrometry analysis of the obtained essential oil was performed using a VARIAN CHROMPACK - CP 3900 system by injection of 0.2 µl of extract. The vector gas was helium, with a debit of 0.3 ml/min. The column used is a capillary column of type CP. Chivasil-Dex CB fused silica WCOTVF5, (25 m, 0.25 mm ID, 0.25 µm film thickness). Oven temperature was programmed (70°C for 2.5 min), and then increased to 280 °C at 15 °C/min, the detector used for this analysis is the type of mass spectrometry (20200 Saturn) with a 250ºC temperature linked by a computer with an appropriate software and a NIST databank to identify the compounds.

Animals and Treatment

The experiences are realized using Wistar albinos rats weighting 200 to 350 g when arriving to the laboratory. The rats are grouped by 3 on Makrolon cages (L x l x H = 40 × 25 × 18 cm) of 2 females VS 1 male, disposed on a ventilated animalery at 21°C ± 1°C. After their arrival, they are used at least after one week. Animals have an adlibitum access to food and water, whereas an artificial illumination establish a day/night cycle (one day between 7 and 19h).On pregnancy day 0, the dams were divided into three groups: one group received 2 g/L lead acetate (Pb $(C_2H_3O_2)_2$) and 4.79 mg/ml manganese chloride tetrahydrate ($MnCl_2$ $4H_2O$) in drinking distilled water during gestation and lactation (Pb-Mn). The second group received Pb-$MnCl_2$ and fennel essential oil (Pb-Mn-FEO), whereas, rats in the control group received distilled water without Pb and $MnCl_2$ (T), as it is previously described. At birth, the Pb-Mn pups continued to receive Pb and $MnCl_2$ during lactation until postnatal day (PND) 21 and the rats were weaned on this day[14,15]. Number and suffering of animals were minimised in accordance with the guidelines of the European Council Directive (86/609/EEC).

Experimental design

Animals were co exposed to lead and manganese during gestation and lactation (Pb-Mn) and the control animals received distilled water. In order to test the ability of fennel essential oil (FEO) to attenuate Pb-Mn toxicity induced, drug therapy was administered, beginning 24 h after weaning. Randomly chosen animals in each group were injected (i.p.) with 0.1 ml/kg of FEO.

Blood samples

At the end of the experiment, the animals were sacrificed in the morning after fasting for 12 h, by IP injection with a solution of chloral ($C_2H_3C_{13}O_2$) to 10%. After incision of the abdomen, blood is collected by cardiac puncture in heparin tubes for biochemical analysis and in EDTA and tubes for hematological evaluation and dry tubes for blood smears.

Biochemical assays
The lead and Manganese concentrations in the blood were determined by using atomic absorption spectroscopy (Perkin-Elmer model 800).

Hematological evaluation
Blood taken with EDTA was used for determination of total red blood cells (RBCs), packed cell volume (PCV), hemoglobin (Hb), mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and platelets count using an automatic cell counter (Sysmex XE-2100, Italy). Blood smears were performed according to standard techniques, after fixation in (alcohol 70%), staining with May-Grunwald-Giemsa and mounted between the slide and the cover slide.

Tissue samples
The bone tissue was carefully removed, rinsed with cold saline and weighed. Then they were fixed in formalin, to be studied by histological techniques.

Statistical Analysis
Results were expressed as mean ± standard error of the mean (SEM). Data were analysed by the two-way analyses of variance (ANOVAs). When a significant difference was found, the Student-Newman-Keuls post-hoc test was conducted. For all analyses, a difference was considered significant at $p \leq 0.05$.

Results and Discussions

Yield of FEO
FEO has been obtained with hydro distillation witha yield of 1, 2 % regarding to the total quantity of the dried material. This is in accordance with research works of El-Sayed et al.[16], who reported yields comprised around 1.52%.

Chemical compositions of fennel essential oil
The analysis of FEO by gas chromatography permitted to identify 14 major compounds cited according to the elution order (Table 1). 14 compounds representing the percentages sum of the obtained compounds has been identified 33.0637% are mono terpene hydrocarbons, 14.9548% are oxygenated monoterpene, 36.0574% of arylpropenesand, 14.2330% of terpene alcohol. 0.3482% of bicyclic sesquiterpene and 0.8378% is a monocyclic sesquiterpene alcohol. The major compounds of this oil are E-anéthole (34.7907%). Our results are consistent with the work of of Rather et al.[17].

Table 1: Chemical composition of fennel essential oil analyzed using GC–MS with concentration percentages of components

Compounds	Retention Time (min)	Concentration (%)
α-pinene	8.95	2.4549
Myrcene	10.54	1.4335
Linalool	11.13	12.792
α-phéllandréne	11.35	1.3325
p-cyméne	11.75	5.1070
Limonéne	11.92	9.5032
Y-terpinéne	12.86	0.4406
Fenchone	13.94	14.0556
Unknown	17.35	14.2330
Estragole	18.93	1.2667
Verbénote	19.13	0.8992
E-anéthole	20.11	34.7907
Unknown	20.43	0.3482
Fenchylalcohol	21.41	0.8378

These compounds concentration varies considerably with physiological state and the fennel origin[9]. The *F. vulgare* essential oil composition exhibits a considerable chemodiversity which depends on the extraction method and geographical origin. The accumulation of these compounds inside the plant is variable, appearing in roots, stem, shoots, flowers and fruits[9]. It was reported that the essential oil content and composition varies during the different maturation stages of *F. vulgare*. The essential oil content was reported to decline with fruit maturity. The trans-anethole, is the main component, varied

between 81.63% and 87.85%[18]. The pharmacological effects of the *F. vulgare* fruits are generally attributed to their essential oil[19].

Body and bone Weights

The exhibition lead acetate and manganese chloride were observed in young Wistar rats which reduced weight body(Table 2). This is due to a decrease in food intake throughout the experiment. Indeed, it was demonstrated that the lead poisoning and manganese induced significant changes in food intake. On the other hand, the works of Smith et al.[20] and Ibrahim et al.[21], showed that lead and manganese provoked an inhibitory effect at the center of the regulation of satiety and hunger which is the hypothalamus.

In addition, administration of *Foeniculum vulgare* (fennel) essential oil by intraperitoneal route in rats exposed to Pb-Mn shows an improvement in the body weight compared to therats intoxicated. This body weight upturn caused by FEO treatment could be attributed to the presence of anethole and estragole, which have digestive stimulating and appetizing effects[16].

Table 2: Body weight (g) and bone weight (g) of control (T) and lead-manganese exposed rats and Pb-Mn treated rats by fennel essential oil (Pb-Mn-FEO) during gestation and lactation

	T	Pb-Mn	Pb-Mn-FEO
Body weight (g)	105.25*	57.21*	82.07*
Bone weight (g)	2.41*	2.16*	2.39*

Data are mean±S.E.M. * p<0.05, (Pb-Mn vs. Control) p<0.05, (Pb-Mn vs. Pb-Mn FEO) .

Biochemical parameters

Lead is not necessary to the life of living beings. After gastrointestinal absorption, lead enters the bloodstream where it is distributed in the erythrocytes, in a non-diffusible form before going to settle in the tissue or be excreted in urine. The blood is the intersection of all paths lead in the body[22]. The atomic spectrophotometry allowed us to register a blood lead level of about 34.1 ± 0.81 µg/dL in Pb rats exposed to 0.2% of Pb acetate (Table 3). These results show a good impregnation of the lead. This means that the blood is a preferential binding sites lead[23], more study Edwards et al.[24], defined the threshold blood lead levels that equal to 10 µg/dL in children. Recent experimental studies have determined the concentration of lead in the inoxicated rats varies between 31.8 and 58.7 µg/dL[25, 26].

Moreover, Mn level found in our study is equal to 16.5 ± 0.08 µg/dL, however previous studies have examined Mn levels in plasma and whole blood, but a few studies have investigated the concentrations of Mn. Thus, they chose Mn levels of erythrocyte as a biomarker to determine the Mn maternal exposures during pregnancy[27]. Other studies have shown that during pregnancy, blood levels of Mn increased during the three semesters and Mn crossing the placenta by active transport[28]. The increase in Mn levels during pregnancy can also be related to the acceleration of erythropoiesis, intestinal absorption or tissue that mobilizes the Mn[29,30].

Table 3: Blood lead and blood manganese concentration (µg/dL) of control (T) and lead-manganese exposed during gestation lactation (Pb-Mn) and treated rats by fennel essential oil

	T	Pb-Mn	Pb-Mn-FEO
Lead concentration (µg/dl)	0.26 ± 0.028*	34.1 ± 0.81*	18.2 ± 2.82*
Manganese concentration (µg/dl)	1.13 ± 0.07*	16.5 ± 0.08*	14.4 ± 0.11*

Data are mean ± S.E.M. * p<0.05, (Pb-Mn vs. Control) *p<0.05, (Pb-Mn vs. Pb-Mn-FEO).

Hematological parameters

The present study demonstrated a significant decrease in RBC count, Hb concentration, and % PCV in Pb-Mn intoxicated rats (Table 4). It induced a microcytic hypochromic anemia. This hematological alteration might be due to the lead effect on aminolevulinic acid dehydratase (ALAD) activity, this is a crucial enzyme of heme synthesis[31]. Moreover, lead inhibits the conversion of coproporphyrinogen III to protoporphyrin IX who trains a hemoglobin production reduction and shortened life span of erythrocytes[32].

Table 4: Effect of co-exposition Pb-Mn and treatment with fennel essential oil (FEO) for 21 days on hematological parameters in rats during pregnancy and lactation period.

	T	Pb-Mn	Pb-Mn-FEO
WBC ×10^3 / mm^3	7.9 ± 6.73 *	1.7 ± 2.29 *	4.68 ± 4.34 *
RBCs×10^6 / mm^3	7.18 ± 0.002 *	4.4 ± 0.04 *	6.2 ± 0.02 *
Hb (g dL^{-1})	14.7 ± 0.05*	7.9 ± 0.05 *	11.9 ± 0.05 *
PCV%	44.2 ± 0.87 *	19.5 ± 0.22 *	32.24 ± 0.65 *
MCV(Fl)	61.5 ± 0.22	44.3 ± 0.048 *	52 ± 2.19 *
MCH (pg)	20.5± 1.8*	17.9± 0.11*	19.2± 0.2*
PLT× 10^3 /mm^3	635.4± 0,21*	465± 7.63*	528.65± 0.97*

Data are mean ± S.E.M. * p<0.05, (Pb-Mn vs. Control) p<0.05, (Pb-Mn vs. Pb-Mn-FEO).

To facilitate the understanding of the pathophysiological mechanisms of lead and manganese intoxication, blood smears were performed. Our results showed shape abnormalities sizes (poikilocytosis), different red blood cells with pale colors characterized by low hemoglobin. There is also an increase in acanthocytes which are characterized by crenellated red blood cells shaped acanthus leaf or spicules in as sea urchin, as we call them echinocytes. Dacaryocytes presence of more decreased number of red blood cells, and platelets (Fig. 1). These abnormalities in the formation and size of red blood cells resulted in microcytic hypochromic anemia go hand in hand with the work of Chang et al.[33], which showed that leads to the inhibition of sulfhydryl-dependent enzymes, such as γ-aminolevulinic acid dehydrogenase and ferrochelatase in heme synthesis. The production of free erythrocyte protoporphyrins can be measured and in fact it's resulted by the disruption of hemoglobin synthesis. In addition, the degradation of ribosomal RNA in red blood cells can be caused by lead inhibition of pyrimidine 5'-nucleotidase. Other study led by Conrad et al.[34], revealed that tissue level Mn can substitute for iron ions in pairs (Fe^{+3}/ transferrin) which is tied to the transmembrane receptors erythroblast (DMT1) and thus accumulated the spinal level.

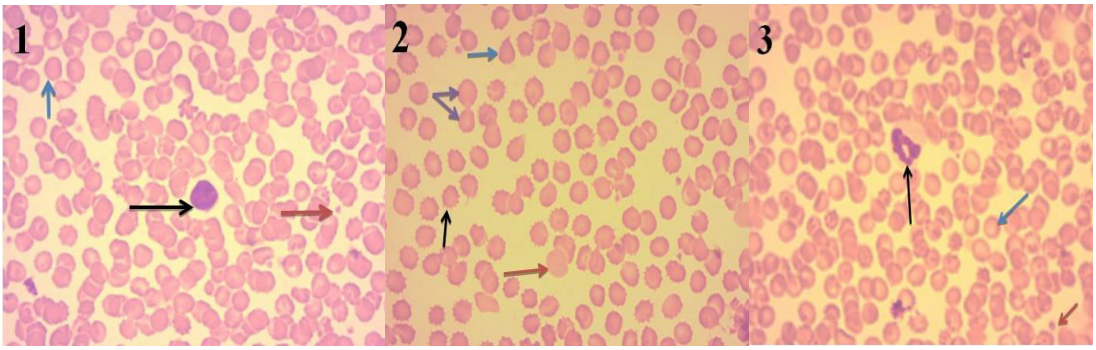

Figure 1: (1)-(3)Blood smear were observed at large magnification (×100), staining with May-Grunwald-Giemsa. A control rats (1)(blue arrow): red blood cells(erythrocytes), (black arrow): basophils, (red arrow): platelets. Rats exposed to Pb-Mn (2) (black arrow): crenellated red blood cells (acanthocyte) (purple arrow): shape anomaly and size of red blood cells (dark blue arrow): dacaryocyte (red arrow): GR with a pale color. Rats treated with FEO (3) (black arrow): Neutrophil (red arrow) platelets (blue arrow): GR normal size

In the same context, the histological examination revealed that exposure to Pb-Mn causes rarification to bone marrow, more adipose involution and a substantial regression of erythroblastosis. These observations confirm the toxic damage induced by lead acetate and manganese chloride on bone and the bone marrow. Our results, then, are consistent with the work of Haleagrahara et al.[6], which indicate that the lead blocks several enzymes necessary for synthesis of hemoglobin and disrupt the calcium binding on hydroxyapatite and bone mineralization[35].

It is possible that the lead has a direct toxic action on bone because over 90% of inorganic lead is fixed in the skeleton. It could increase bone loss by inhibiting the activation of vitamin D, decreasing calcium uptake, and also by interfering with the hormonal regulation of bone metabolism[36]. This can be a total of more than 200 mg per individual. Its half-life in bone is very long, with the order of 1 year in trabecular bone and from 10 to 20 years in the compact bone. Thus, the bone shows an integrator of the exposure past the lead seat of the accumulation of toxic. This inhibits the production of

astéocaline by osteoblasts, and after absorption by the hydroxypathie through displacement of these calcium binding sites on the ostéocaleine[37].

El-Ashmawy et al.[38], reported that lead toxicity causes a weak clastogenicity effect on rat bone marrow cells. They observed a reduced dividing cells number, increased abnormal cells number and an increased chromosomal aberrations frequency. The observed change could be due to the excessive ROS generation or failure of the cellular antioxidant system[39].According to Pejovic-Milic et al.[40].Mn ions accumulated in bone tissue. This accumulation in the bones may have a direct detrimental effect on bone's normal structure and function. In addition, the work of Lan Hong et al[41], reveals that the Mn positively associated with Fe and Zn in bone but inversely related to Cu. However, the implications of these relationships are currently unknown.

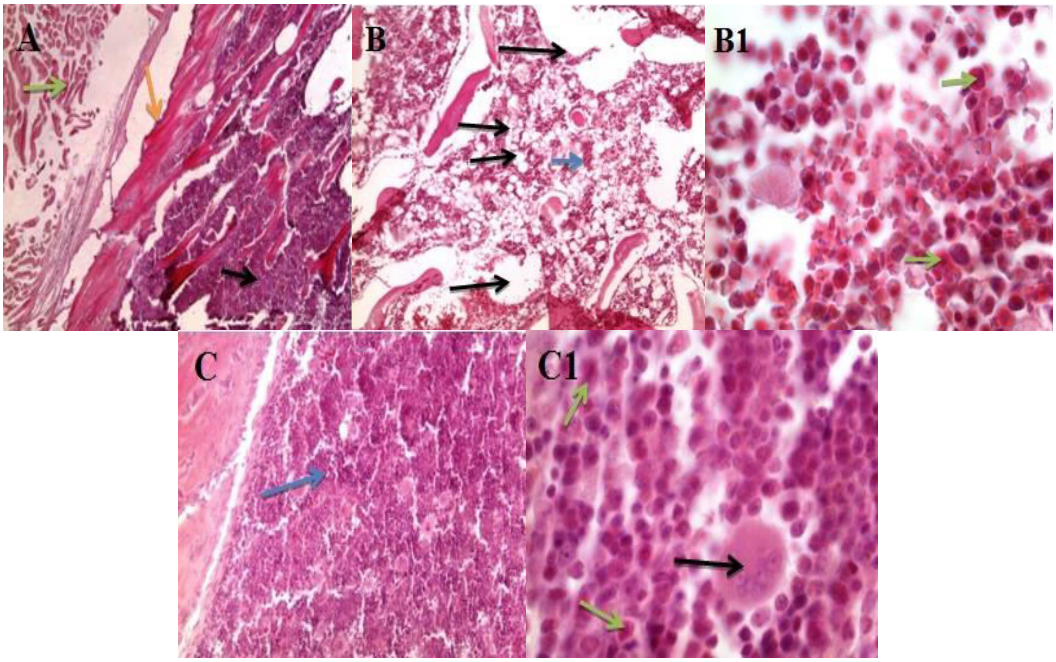

Figure 2: Light microscopic views of a bone tissue stained with hematoxylin and eosin (A) Control rats, (A) G (x 5), black arrow: bone marrow, orange arrow: Bone, green arrow: striated muscle.(B, B1, B2) Pb-Mn intoxicated rats, (B)G (x 5), black arrow: adipose involution, blue arrow: therarification bone marrow. (B1) G (x 100), green arrow: erythroblastic. (B2) G (x 40), black arrow: Adipose involution. (C, C1) Rats intoxicated and treated with FEO, (C) G (x 20), blue arrow: improving the bone marrow (the presence of erythroid). (C1) G (x 100), green arrow: erythroblastic, black arrow: megakaryocyte.

After FEO treatment the exposed rats show a hematological parameters upgrade. Our results agree with those of Mansouri et al.[42],they indicated that fennel extract increased significantly the RBCs number in vehicle rats by their antioxydant properties. Some studies have shown that phenolic compounds isolated from fennel aqueous extracts have an absorption and a removal activity of free radicals. The pharmacological effects of this plant can be attributed these compounds and their antioxidant effects [43]. Study of Oktay et al.[44],showed that fennel seeds aqueous and ethanolic extracts have the strongest antioxidant effects according to the absorption tests of anionic radicals and chelating metals activity. Therefore, fennel antioxidant activity maintains the RBC membrane against oxidizing factors and increases their life-time. Anethole has antioxidant and anti-inflammatory effects and wound healing activity in experimental models. Therefore, there is a significant increase in WBC treated by fennel seeds extracts[45]. While, the essential oil of fennel rats addicted to Pb-Mn we have noticed an improvement in blood smears treated rats compared with controls due to the presence of D-limonene and Mycenae which have antioxidant properties[42].

Conclusion

Our study revealed that both of Pb-Mn exposure during developmental period caused hematological and bone marrow architecture changes. In addition, our data showed that *Foeniculum vulgari*

essential oil has an ability to protect bone marrow from damaging effects of lead and manganese toxicity.

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